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14. ABSTRACT The goal of the current study is to identify the molecular mechanisms that contribute to TBI or AD-related impairment so that this information can then be used to identify at-risk individuals and develop effective therapeutic approaches. Specifically we will test the hypothesis that level of protein phosphatase 2A (PP2A) methylation affects sensitivity to behavioral impairments resulting traumatic brain injury caused by shockwave exposure. The primary bases for this hypothesis are the observations that 1) aggregates of hyperphosphorylated tau are a common feature of multiple neurodegenerative conditions including Alzheimer's disease and traumatic brain injury-associated degeneration, and 2) that PP2A is the principal tau phosphatase. To test this hypothesis, we generated two lines of transgenic mice, one that over expresses the PP2A methylesterase, PME, and a second that over expresses the PP2A methyltransferase, LCMT. In previous work we found that transgenic PME over expression sensitized animals to electrophysiological and behavioral impairments caused by exogenous beta-amyloid exposure and that LCMT over expression protected animals from these impairments. Our prediction is that over expression of these transgenes will exert similar effects with respect to shockwave-induced impairments. In the past year of the project, we identified shockwave exposure conditions that reliably elicit acute increases in tau phosphorylation in mice, and assessed the effect of PME over expression on these increases. We also identified shockwave-induced behavioral impairments in these animals and similarly assessed the effect of PME over expression on these impairments. We also conducted a preliminary histological analysis of eyes from shockwave-exposed mice that identified shockwave related tissue damage at 24 days after injury. Additional, planned behavioral and histological analyses are ongoing that will allow us to complete our test of the hypothesis and provide new insights into the molecular mechanisms that control sensitivity to TBI-related impairments.

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INTRODUCTION:

Neurodegeneration resulting from both traumatic brain injury (TBI) and Alzheimer's disease (AD) is characterized by aggregates of hyperphosphorylated tau (Ballatore et al., 2007; Dekosky et al., 2013). This observation together with the involvement of tau in other neurodegenerative disorders suggests that a common neurodegenerative mechanism involving tau hyperphosphorylation may contribute to impairments associated with both TBI and AD. Tau phosphorylation is controlled by a balance between the activity of numerous kinases and the protein phosphatase, PP2A (Martin et al., 2013), and PP2A activity is in turn controlled by C-terminal methylation of its catalytic subunit (Sents et al., 2013). To examine the effects of altered PP2A activity and tau phosphorylation on TBI and AD-related impairments, we generated two lines of transgenic mice, one that expresses the PP2A methylesterase, PME-1, and one that over expresses the PP2A methyltransferase, LCMT-1. We found that PME-1 over expression increased sensitivity to electrophysiological and behavioral impairments caused by acute oligomeric Aβ exposure, and that LCMT-1 over expression protected animals from these impairments. In this project, we are using these novel transgenic animals to examine the relationships between shockwave exposure, tau phosphorylation, and behavioral impairment. If tau phosphorylation is involved in injury-induced and AD-related impairments, then we expect that these transgenes will exert similar sensitizing or protective effects on shockwave-induced impairments.

BODY:

In June of 2013 the statement of work for this project was modified to address the need for a more comprehensive assessment of the link between the shockwave characteristics and exposure conditions, and injury related increases in tau phosphorylation. The current statement of work seeks to address 4 main questions:

1) **What are the parameters and characteristics of shockwave exposure that are necessary to produce increased tau phosphorylation in mouse brain?**
2) **What are the consequences of LCMT-1 and PME-1 transgene expression on bTBI-associated behavioral impairment and tau phosphorylation at 2 weeks and 3 months post injury?**
3) **Do LCMT-1 and PME-1 transgene expression alter the acute shockwave-induced changes in tau phosphorylation at 1 hr and 24 hrs post-injury?**
4) **Do LCMT-1 and PME-1 transgene over expression affect tau hyperphosphorylation induced by acute beta-amyloid exposure?**

In the past year we have made rapid and substantial progress in addressing these questions as detailed below.

Shockwave exposure conditions: In the past year, we successfully identified shockwave exposure protocol that produces acute increases in tau phosphorylation. This protocol consisted of a single shockwave exposure of 269 ± 9.8 kPa peak over pressure, 0.73 ±

0.021 ms duration, and 67 ± 2.3 kPa-ms impulse directed at the top of the head under conditions where head is supported from below and acceleration was limited. As shown in **Figures 1-4**, this protocol elicits increases in tau phosphorylation of at least 3 epitopes that can be detected in both hippocampal and cortical homogenates at 1 and 24 hours post-injury (see **Figure 5**).

These data support the contention that traumatic brain injury can cause alterations in tau phosphorylation that may contribute to neurodegeneration, and that this process can be modeled effectively in mice.

Effect of PME over expression on shockwave-related tau phosphorylation: We tested the effect of PME over expression on shockwave-induced increases in tau phosphorylation at both 1 and 24 hrs after injury. We found that while PME over expression correlated with increased basal levels of tau phosphorylation at specific sites, it did not increase further the shockwave-induced tau phosphorylation we observed in sibling controls (**Figures 1-4**). These data suggest that the effect of shockwave exposure on tau phosphorylation may reach saturation at the sites where we observe increases at 1 and 24 hrs post-injury.

We are currently in the process of testing whether PME over expression may affect the persistence of these shockwave-induced increases in tau phosphorylation. Our initial analysis of tau phosphorylation in PME over expressing and control animals 24 days after shockwave exposure suggests that phosphorylation in both these groups returns to baseline by this time point (**Figure 5**), and we are in the process of confirming this result. This analysis will also be carried out on homogenates prepared from animals 3 months after shockwave exposure, and we are in the process of collecting the tissue for these experiments as animals reach this post-injury time point.

LCMT over expression is predicted to promote PP2A methylation and tau dephosphorylation. To explore the possibility that LCMT over expression may protect against shockwave-induced increases in tau phosphorylation, we exposed these and control animals to shockwaves or sham treatments and harvested brain tissue at 1 and 24 hrs after injury. In the past year, we completed blast exposure and tissue harvesting for this experiment and western blot analysis of tau phosphorylation is now in progress. If we find that LCMT over expression does reduce shockwave-induced tau phosphorylation, this would identify LCMT and/or PP2A as potential therapeutic targets for preventing TBI-related neurodegeneration and potentially other tauopathies.

Shockwave-related behavioral impairment: During the past year, we completed our behavioral analysis of PME over expressing and control animals at the planned 2-week (13-23 day) post-injury time point. We found that shockwave exposure affected the performance of mice in both a novel open field and in a radial arm water maze task.

In the open field environment, shockwave-exposed mice spent a significantly higher proportion of time in the center of the arena (**Figure 12**). This measure is commonly used as an index of anxiety, and therefore suggests that shockwave exposure may blunt the anxious response of mice to novel environments. This behavioral change was accompanied by non-statistically significant trends for increased activity in the anxiogenic open field and elevated plus environments (**Figure 16**), for increased open arm time in the elevated plus apparatus (**Figure 18**), and for reduced baseline (pre-shock)

freezing in the contextual fear conditioning apparatus (**Figure 21**). None of these shockwave-related differences were affected by over expression of PME.

In the 2-day radial arm water maze task, shockwave exposed animals committed significantly more errors in navigating to the hidden platform during the second day of this task (**Figure 23-24**). This task is typically used as a measure of hippocampus-dependent short-term spatial memory and has been found to be sensitive to genetic manipulations in mouse models of Alzheimer's disease. It is also a task that we used previously to show alterations in A-beta sensitivity in the PME and LCMT transgenic mice. The impairment that we observe here suggests that shockwave exposure leads to cognitive impairment that persists for up to two weeks after injury. Like the acute shockwave-induced increases in tau phosphorylation, PME over expression does not appear to affect the magnitude of this cognitive impairment at this time point.

In a visual platform water maze task, we found that shockwave exposure produced a deficit in PME over expressing animals that was not observed in injured controls (**Figure 25**). Performance of this task requires a simple non-spatial association between a visual cue and an escape platform. Neither transgene expression nor shockwave exposure affected swimming speed suggesting comparable motivation and motor performance among these groups. This selective impairment, therefore, suggests that PME over expression may sensitize some portion of the visual system to the effect of shockwave exposure.

These animals were also tested in a rotarod task to assess motor function (**Figure 10-11**), and in forced swim and tail suspension tasks to assess the effect of shockwave exposure and PME over expression on affect (**Figure 27-28**). A contextual fear-conditioning task was also carried out on these animals as a second measure of cognitive performance (**Figure 21**) along with a sensory threshold measure to test for potential differences in shock perception in this task (**Figure 22**). We detected no significant differences among the shockwave or sham treated, PME or control groups in any of these tests.

We are in the process of conducting this battery of behavioral tasks on PME and control animals 3 months after shockwave or sham exposure, as well as on LCMT and control animals at both 2 weeks and 3 months after shockwave or sham exposure. Our progress in completing these experiments is detailed in the table below.

	Transgenic line	
Post-injury time point	**PME**	**LCMT**
2 weeks	Testing completed	Pretraining and shockwave exposure complete for 2 of 5 cohorts
3 months	Pretraining and shockwave exposure completed; behavior testing completed on 2 of 5 cohorts	Pretraining and shockwave exposure complete for 1 of 5 cohorts

Blast-related histological changes:

Since visual impairment is a common comorbidity for TBI (Jacobs & Van Stavern, 2013), and eye damage following blast exposure has been reported in several animal models {Hines-Beard, 2012 #1111;Yan, 2009 #1109;Sherwood, 2014 #1099;Bricker-Anthony, 2014 #1098}, we decided to histologically examine eyes from our shockwave exposed animals to assess the presence of eye damage that may affect behavioral performance. To do this we entered into a collaboration with colleagues at Columbia University and the University of Iowa.

We fixed and embedded eyes harvested from animals 24 days after shockwave, sectioned and stained with H&E and looked for evidence of tissue damage. We found that shockwave exposure lead to detectable hematoma and retinal detachment in 3 out of 6 eyes examined from shockwave-exposed mice (**Figure 29-31**). These observations raise the question of whether and to what extent shockwave-induced eye damage may affect behavioral performance in this and other rodent models of blast exposure. In our experiments, it is possible that eye damage may contribute to the apparent reduction in anxiety in the novel open field test (and trends in the elevated plus and contextual fear conditioning task), and could also contribute to the impairment we observe in the radial arm water maze. However, eye damage is unlikely to form the basis for the deficit we observe in the visible platform water maze task since this effect was only observed in shockwave exposed PME mice and not in shockwave exposed controls.

We plan to examine more eyes from this and later time points and correlate any damage we observe with any contemporaneous behavioral deficits. In the longer-term, determining whether and to what extent this damage may affect behavioral performance will require more sensitive measures of visual acuity (eg. electroretinogram, visually evoked potential recordings, optomotor tests of visual acuity) and this analysis will be important for the development , refinement, and interpretation of future rodent and animal models of blast exposure.

We are also in the process of histologically examining brain tissue from shockwave and sham exposed animals. To do this, we are staining paraffin embedded sections with H&E stain to examine general anatomical and cellular morphology, and performing immunohistochemistry using anti-APP antibody, the phospho-tau antibody AT8, anti-phospho-neurofilament light chain (Nfl-1) antibody, anti-glial fibrillary acidic protein (GFAP) antibody – a marker of astrocyte activation, and anti-ionized calcium binding protein-1 (Iba-1)- a marker for microglia activation. To date the majority of our efforts have been directed at optimizing staining conditions for these antibodies, but we now have stained sections from control and PME over expressing animals exposed to shockwave or sham treatment and harvested at 1 hr, 24 hrs, or 24 days after injury. Preliminary analysis of H&E stained sections revealed no gross cellular or anatomical changes resulting from shockwave exposure at these time points in either controls or PME transgenic animals. Analysis of slides stained by immunohistochemistry is in progress.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified a shockwave exposure protocol that produces acute increases in

tau phosphorylation
- Completed planned measures of tau phosphorylation in PME over expressing and control animals at the both the 1 and 24 hr post-exposure time points
- Completed shockwave exposure and tissue harvesting at 1 and 24 hrs post-exposure for the target number of LCMT over expressing, and control animals for the biochemical measures of tau phosphorylation
- Completed planned behavioral analysis of PME over expressing and control animals at the 2-4 week post-exposure time point
- Conducted preliminary histological analysis of eyes from mice that revealed hemorrhage and retinal detachment 24 days after shockwave but not sham exposure.
- Made progress toward behaviorally, biochemically and behaviorally testing the target number of, PME over expressing, LCMT over expressing, and control animals for the remaining 2 week and 3 month post-exposure time points.

REPORTABLE OUTCOMES:

- There were no new reportable outcomes for this reporting period.

CONCLUSION:

We have successfully identified a methodology that recapitulates blast-induced increases in tau phosphorylation and behavioral impairments in mice, establishing a useful model in which to examine the molecular mechanisms underlying TBI-related impairment and neurodegeneration. We are currently in the process of using this model to address the question of whether or not alterations in PP2A methylation or activity may affect the sensitivity to these impairments, however our initial observations suggest this protocol may produce saturating effects with respect to acute tau phosphorylation and behavioral impairments at 2 weeks that are not further enhanced by PME over expression. With the completion of a similar analysis on PME over expressing animals at 3 months post-injury, we hope to test whether this transgene may affect the persistence or progression of these changes. Parallel analyses are in progress on the LCMT over expressing animals to test the prediction that this transgene may reduce the biochemical and behavioral effects of shockwave exposure. Our preliminary histological analysis of eyes from shockwave-exposed mice that identified shockwave related tissue damage at 24 days after injury, raising the possibility that shockwave-induced eye damage may affect behavioral performance – a possibility that has been largely ignored in previous studies of the behavioral effects of shockwave exposure in rodents. Thus far this work represents significant progress toward addressing the immediate goal of understanding the role of PP2A and tau phosphorylation in behavioral impairments, as well as the broader goal of developing effective animal models of blast-related neurodegeneration.

Methods:

Shockwave exposure:

We exposed animals to shockwaves with peak overpressures of approximately 267 ± 7 kPa (mean ± SEM), durations of 0.662 ± 0.009 ms, and impulses of 53 ± 0.9 kPa*ms. According to the Conventional Weapons Effects Program (CONWEP), this level of blast severity can be considered approximately equivalent to exposure to an explosion experienced 0.8 m away from 90 g of C4 explosive (unscaled duration), which is within a range of realistic blast threats in a military setting. With exposure duration scaled to the mouse, according to scaling laws used for pulmonary blast injury, this level is comparable to exposure to an M117 bomb (222 kg TNT) at close range. These animals were placed in the specially designed animal holder described above that supported the head from below, and shielded the body to prevent confounding lung/bowel injury and to reproduce the presence of body armor. In these experiments, shockwaves were directed at the head from above. These conditions produced no lethality in any of the animals tested.

Western blotting:

Phospho-tau levels were determined by western blot on brain homogenates prepared by sonication in hot 3%LDS/50 mM Tris pH 7.5/10 mM EDTA. Proteins were resolved on 4-12% Bis-Tris protein gels, blotted on PVDF membranes and probed with the indicated primary antibodies. Detection and quantification was carried out using Licor infrared-dye labeled secondary antibodies and an Odyssey imager. In all cases band intensities were normalized to a within-lane control band (either total tau or β-actin).

Behavioral testing:

The behavioral protocols were carried out according to the following schedule:

Days -4 to -1: Rotarod pretraining

Day 0: no testing (shockwave exposure day)

Battery Day 1: openfield (AM) and accelerating rotarod (PM) (for experiments on transgenic animals this day will be 2 weeks, or 3 months after shockwave exposure)

Battery Day 2: elevated plus maze (AM) and forced swim test (PM)

Battery Day 3 and 4: radial arm water maze task

Battery Day 5: tail suspension test (AM) and contextual fear conditioning task training (PM)

Battery Day 6 : contextual fear conditioning task testing

Battery Day 7 and 8: visible platform water maze task

Battery Day 9: sensory threshold assessment

Accelerating rotarod task:

We assessed motor performance of mice using a rotarod apparatus (Med Associates) essentially as described previously (Clausen et al., 2011; Wang et al., 2011; Yu et al., 2012). This apparatus consists of a 32 mm diameter rotating rod suspended 16.5 cm above a pressure sensitive tray. The rod passes through large plastic discs that create 57 mm lanes along the rod in which lateral movement of the mice are constrained.

Training on this task was carried out on 4 successive days. The first day of training consisted of 4 x 5 minute trials. On the first trial, animals were placed on the apparatus and the rotation speed was set at 4 rpm, on the second and third trials, the rotation speed was slowly ramped up from 4 to 10 rpm over the course of the trial, and on the third trial the rotation speed was ramped from 4 to 40 rpm. On this first day of training, animals that fell were returned to the rod and the trial continued for the specified 5 min period. On this and all subsequent days, animals were returned to their home cages for 45 min between trials. The second through fourth days of training consisted of 3 x 5 min trials per day with the rotation speed ramped from 4 to 40 rpm over the course of the trial. When animals fell from the apparatus the trial was terminated and the animal returned to its home cage. Rotarod testing was carried out in the morning of the second day of the behavioral battery. Testing consisted of 4 trials conducted in the same manner as described for pre-training days 2 -4.

As shown in Figure 6, this pilot study produced a level of performance consistent with similar studies described in the literature that is suitable for detecting motor deficits in our shockwave-exposed animals. The fact that the performance of our animals did not improve across training trial and failed to reach a plateau as high as that reported in some studies is of some theoretical, if not practical, concern. We are therefore repeating this test on a second group of control animals using a different rotarod apparatus to ascertain if these results were a function of the particular apparatus used for the initial tests.

Open field testing:

To assess the effects of shockwave exposure and our genetic manipulations on activity level, response to novelty, and anxiety, we will assess the behavior of our animals in a novel open field environment essentially as described in (Tweedie et al., 2007). In our pilot experiment, we placed animals in a plexiglass chamber (43.2 cm long × 43.2 cm wide × 30.5 cm high) for a total of 30 min during which time their movements were tracked and analyzed using a video tracking system and behavioral analysis software (Ethovision, Noldus). This analysis revealed levels of activity (ambulatory distance) and thigmotaxis (center time) (Figure 7) that were consistent with published literature and suitable for assessing the effects of shockwave exposure and transgene expression in our planned experiments.

Elevated plus maze task:

To assess any possible anxiogenic or anxiolytic effects of shockwave exposure and our genetic manipulations, we will examine the behavior of our animals in an elevated plus maze essentially as described in (Schwarzbold et al., 2010; Siopi et al., 2012). The apparatus consists of a plus shaped track with arms 18 cm long and 6 cm wide, elevated 60 cm above the bench top by a single central pillar. Two non-adjacent arms are surrounded by walls on 3 sides, and the remaining two arms are exposed. Animals were placed into the center of the apparatus and the number and duration of open vs. closed arm entries are used as an index of anxiety. Animal location during single 5 min exposure to this behavioral apparatus was monitored and analyzed using a video tracking system and accompanying behavioral analysis software (Ethovision, Noldus). After each trial, animals were returned to their home cages and the apparatus was thoroughly cleaned and deodorized with MB-10 and distilled water. We found that

control animals showed strong preference for the closed vs. open arms of the maze spending 19 and 52% of their time in these locations respectively. This level of performance is consistent with published literature and appropriate for detecting differences in anxiety levels in our genetically modified, shockwave-exposed animals.

Forced swim test:

To assess the effects of shockwave exposure and our genetic manipulations on depressive behavior, we assessed the behavior or our animals in forced swim test essentially as described in (Milman et al., 2008; Tweedie et al., 2007). In our pilot experiment, we placed control animals into a 4 liter plastic beaker filled half way with tap water (22-25C) for a total of 6 minutes. During this time, the animals' movements were recorded using a video camera, and the recordings were subsequently offline by a blinded observer for number, timing, and duration of periods of immobility. Following the forced swim trial, animals were dried using paper towels and returned to clean home cages partially illuminated by a heat lamp for a period of 10 minutes to prevent hypothermia. As shown in Figure 8, our pilot study produced a level of performance consistent with published literature and suitable for detecting differences in depressive behavior in our genetically modified, shockwave-exposed animals.

Radial arm water-maze task:

To assess the effects of shockwave exposure and our genetic manipulations on cognitive performance, we will test our animals in a 2-day radial arm water-maze task as described previously (Goldstein et al., 2012). The test will be performed in a 120 cm diameter pool containing a 6-arm radial maze insert and opaque water maintained at 24°C. On each day of the task, animals are subjected to a total of 15 trials. During the first 11 odd-numbered trials of the first day, the location of the escape platform is indicated by a marker protruding above the surface of the water, while on all other trials, the submerged platform is not visible to the animals. In each trial, the number of errors (entries into arms that do not contain the platform) will be recorded. At the end of testing, the mice will be dried off and placed in a clean cage with extra paper towels to prevent hypothermia.

We used this task previously to demonstrate increased sensitivity and resistance to Aβ-induced cognitive impairments in our PME-1 and LCMT-1 transgenic mice respectively (see Appendix 3). In our pilot experiment, conducted on control animals in the same genetic background, we noted a more rapid acquisition of this task and a higher level of performance once acquired (Figure 9) than what we observed previously. We attributed this to the more extensive history of behavioral testing and handling that animals experience when subjected to our behavioral battery and our concern was that the more rapid acquisition may be less sensitive for detecting the protective or sensitizing effects of our transgenic manipulations. To compensate, we reduced the number of visible platform trails used in our protocol from 7 to 2, and conducted a second pilot experiment on another group of control animals, but found that this had a negligible effect on acquisition (Figure 9).

While it may be possible to further adjust the behavioral protocol to achieve a more gradual increase in spatial memory acquisition (for example by substituting an abbreviated version of a traditional hidden-platform Morris water maze for the radial

water maze task we currently use), we believe that this is unnecessary. The existing 2-day radial arm water maze task was successful in detecting transgene dependent alterations in Aβ-induced cognitive impairment and is therefore an excellent candidate for detecting similar transgene-dependent alterations in shockwave-induced cognitive impairment. Its use for the shockwave experiments, also allows a more direct comparison with our data on the effects of these transgenes on sensitivity to Aβ-induced cognitive impairment.

Tail suspension test:

As a second test of the effects of shockwave exposure and our genetic manipulations on depressive behavior, we will assess the behavior or our animals in a tail suspension test essentially as described in (Schwarzbold et al., 2010). In our pilot experiment, animals' tails were gently taped approximately 2 cm from the end to a horizontal bar elevated 30 cm above the benchtop. The animals were then suspended in this position for 6 minutes while their movements were recorded using a digital video camera. Videos were later scored offline by a blinded observer for number, timing, and duration of periods of immobility. Immediately after testing animals were removed from the apparatus returned to their home cages. As shown in Figure 10, our pilot study produced a level of performance consistent with published literature and suitable for detecting differences in depressive behavior in our genetically modified, shockwave-exposed animals.

Contextual and cued fear conditioning:

As a second test of the effects of shockwave exposure and our genetic manipulations on cognitive performance, we will test animals on a contextual fear conditioning task as described previously (Francis et al., 2009; Puzzo et al., 2008). In this task, animals are placed into a conditioning chamber located inside a sound-attenuating box (72cm x 51cm x 48cm). A clear Plexiglas window (2cm thick, 12cm x 20cm) will allow the experimenter to record the animal's behavior with a video camera connected to a computer running Freeze Frame software (MED Associates Inc.). Background white noise (72dB), will be provided by a single computer fan will installed in one of the side of the sound-attenuating chamber. The conditioning chamber (33cm x 20cm x 22cm) is made of transparent Plexiglas on two sides and metal on the other two. One of the metal sides has a speaker and the other one a 24 V light. The chamber has a 36-bar insulated shock grid floor. The floor is removable to facilitate its cleaning with MB-10 and then with distilled after each experimental subject. Animals will be placed in the conditioning one animal at a time chamber once on each of two consecutive days. The first day of exposure mice will be placed in the conditioning chamber for 2 minutes before the onset of a discrete tone (CS) (a sound that will last 30s at 2800Hz and 85dB). In the last 2s of the CS, mice will be given a foot shock (US) of 0.50mA for 2s through the bars of the floor. After the tone and shock exposure, the mice will be left in the conditioning chamber for another 30s and then placed back in their home cages. 24 hours after their first exposure animals will be returned to the conditioning chamber for a total of 5 min without foot shock or tone presentation. During each of these exposures, freezing behavior will be scored using FreezeFrame software (Med Associates) and this parameter will be used as a measure of the strength of the context-shock association (ie. memory on the second exposure) and the general level of anxiety (baseline pre-shock exposure).

We found that this protocol produced low levels of baseline freezing in our control animals prior to shock exposure (7% freezing) that increased dramatically during reexposure to the shock context 24 hours later (50% freezing). This level of performance is consistent with our previous experiments and published literature, and is appropriate for detecting differences in contextual learning and memory performance in our genetically modified, shockwave-exposed animals when included as part of our behavioral battery.

Sensory threshold assessment:

As part of our pilot experiment, we also tested animals on the sensory threshold assessment task that we will to use to rule out any differences in shock perception that could interfere with our interpretation of the performance of animals in the contextual fear conditioning task. We conducted this assessment as described previously (Francis et al., 2009; Puzzo et al., 2008). Animals were placed into an apparatus similar to that used for contextual fear conditioning. A sequence of single, 1sec foot shocks was then given at 30 sec intervals and 0.1 mA increments from 0 to 0.7 mA. Each animal's behavior was evaluated to identify shock intensities that produced the first visible response to the shock (flinch), the first extreme motor response (run/jump), and the first vocalized distress (Figure 11). This analysis produced results similar to data we obtained previously and to published results.

Visible platform water maze task:

To complete the pilot testing of our behavioral battery, we subjected our cohort of control animals to a visible platform water maze task as described previously (Francis et al., 2009; Puzzo et al., 2008). We will use this task in our behavioral battery both as another assessment of motor function and also to test for any performance deficits that might interfere with our analysis of the radial arm water maze task. We performed this task in the same 120 cm diameter pool used for the radial arm water maze task, except that the partitions were removed. Training for this task was carried out over 2 days with 3 morning and 3 afternoon trials on each day. Intertrial intervals were 15 to 20 min and rest periods between morning and afternoon sessions were at least 3 hrs. Each trial lasted for a maximum of 120 sec during which animals were required to swim to a visible escape platform located just above the water surface. Animals that did not reach the platform within the allotted time were guided to it and allowed to sit there for 15 sec before being returned to their home cage. The location of the platform was rotated among 4 different locations such that it was not be present in the same location on any two successive trials. Water temperature was maintained at approximately 24°C, and at the end of testing, the mice were dried off and placed in a clean cage with extra paper towels to prevent hypothermia. Measures of both time required to reach the hidden platform (latency) and swim speed (Figure 12) were conducted using a video-tracking system and behavioral analysis software (Ethovision, Noldus), and were consistent with the previous data from these animals and published literature.

Histology:

Eyes: Eyes: Eyes were removed by blunt enucleation from mice sacrificed by cervical dislocation. Eyes were the placed in acidic methanol solution before paraffin embedding and sectioning. Sections were then processed with hematoxylin and eosin, and standard images were captured under light microscopy for review.

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SUPPORTING DATA:

Figure 1: tau phosphorylation in cortex of control or PME over expressing animals 1 hour after shockwave exposure or sham.

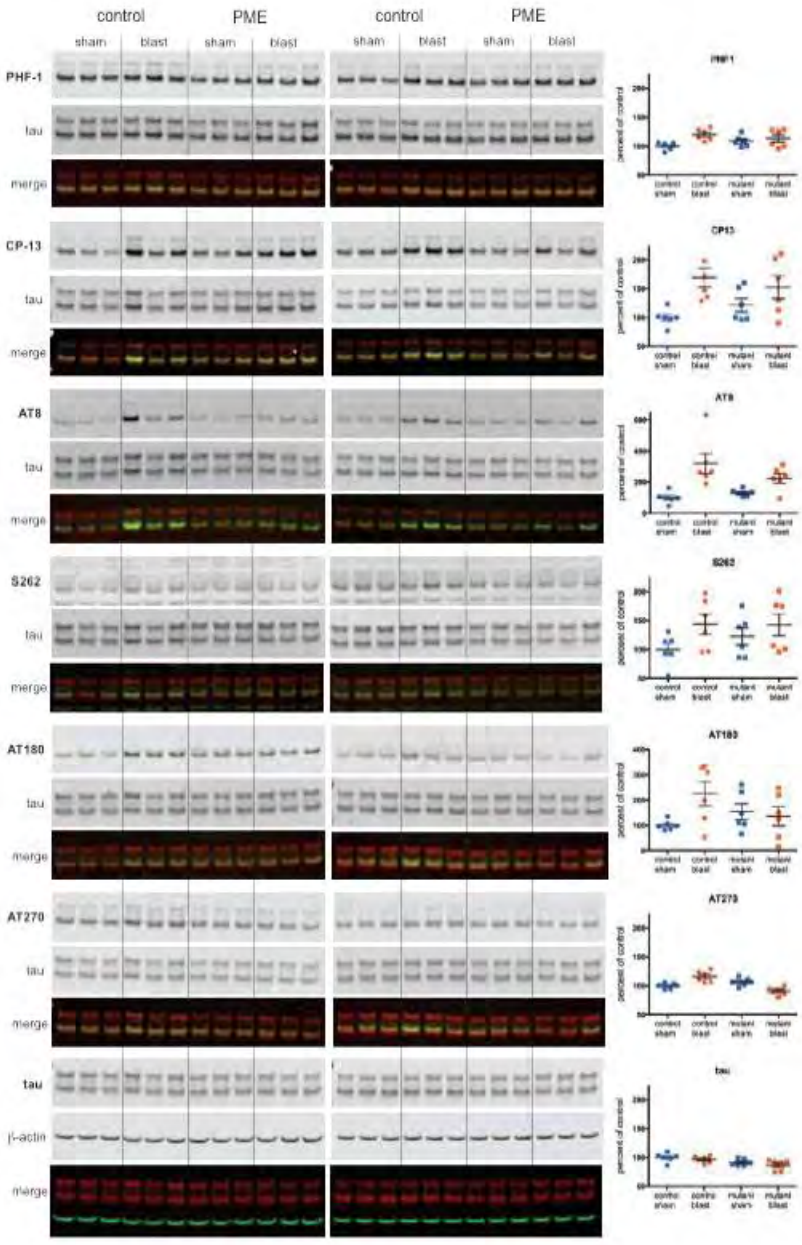


Figure 2: tau phosphorylation in cortex of control or PME over expressing animals 24 hours after sham or shockwave exposure.

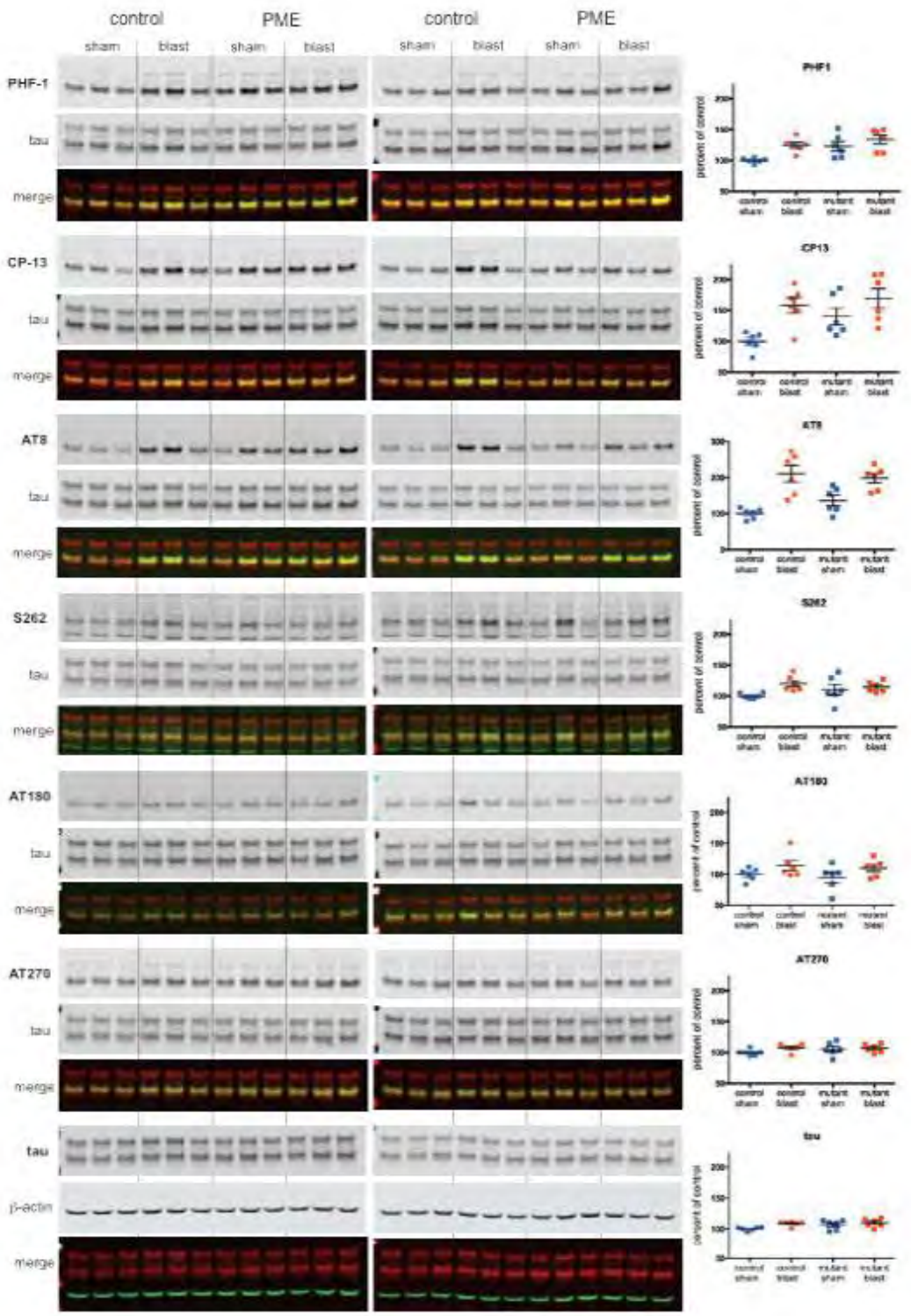


Figure 3: tau phosphorylation in hippocampus of control or PME over expressing animals 1 hour after shockwave exposure or sham.

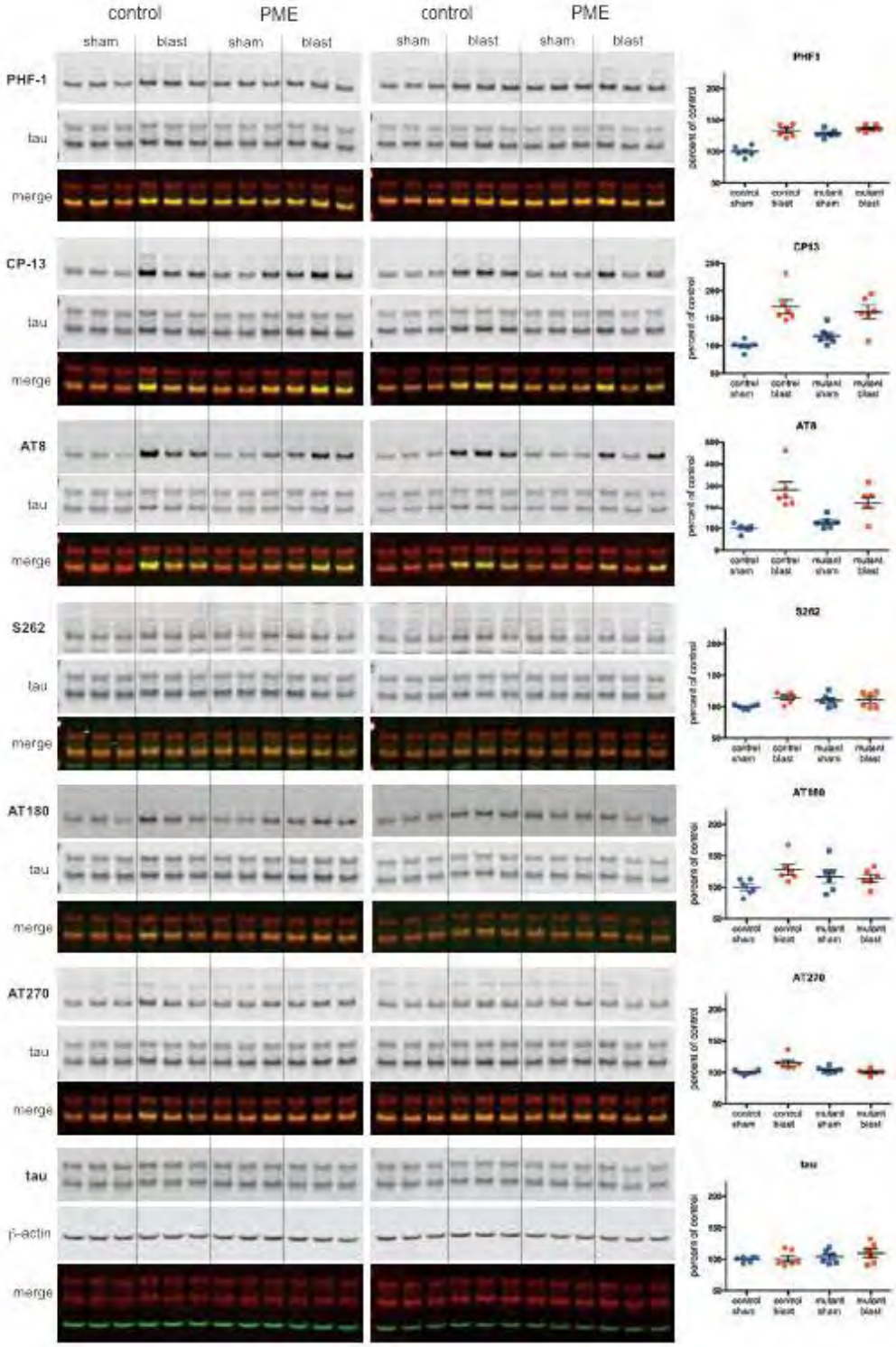


Figure 4: tau phosphorylation in hippocampus of control or PME over expressing animals 24 hours after sham or shockwave exposure.

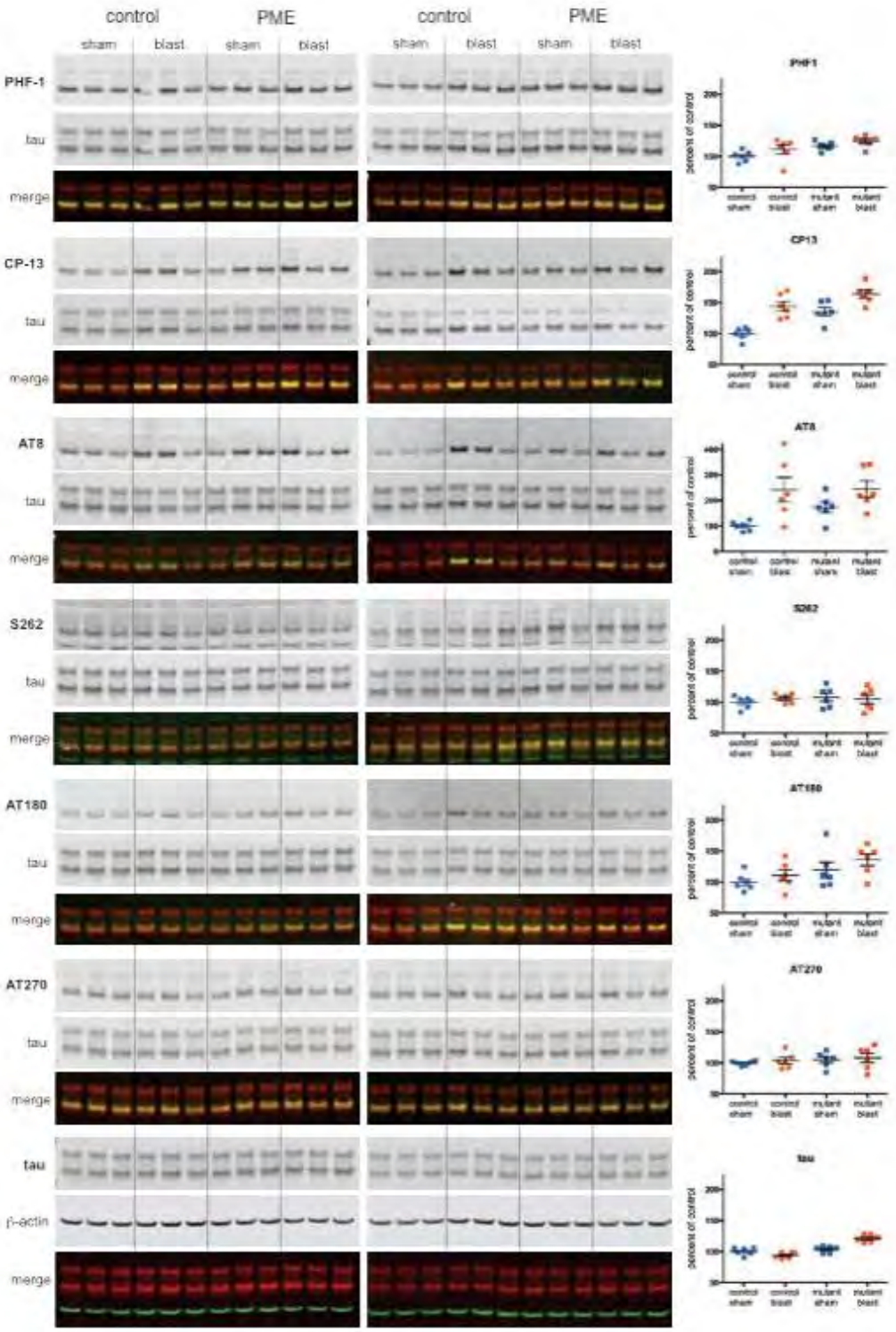


Figure 5: tau phosphorylation in hippocampus of control or PME over expressing animals 4 weeks after sham or shockwave exposure.

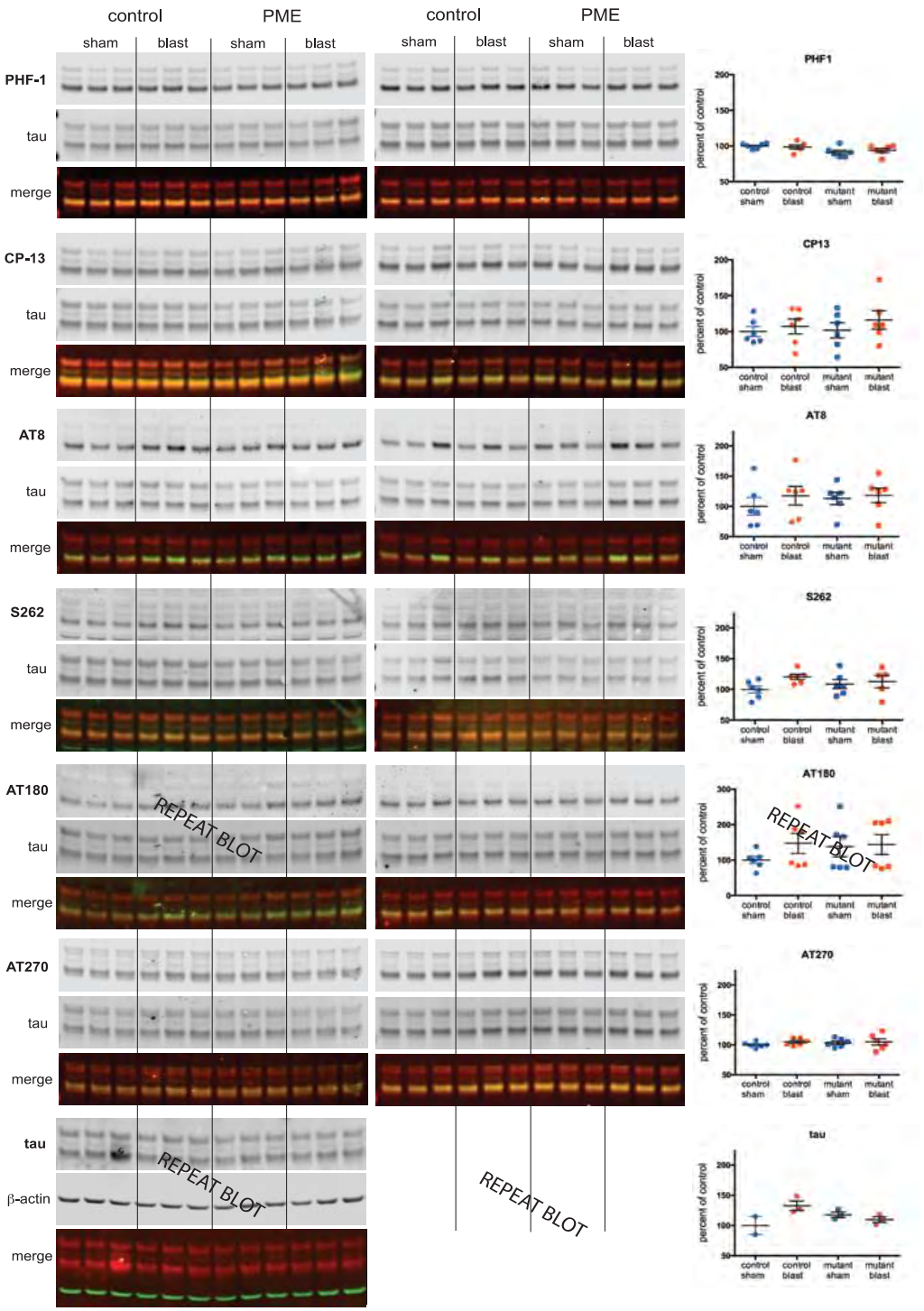
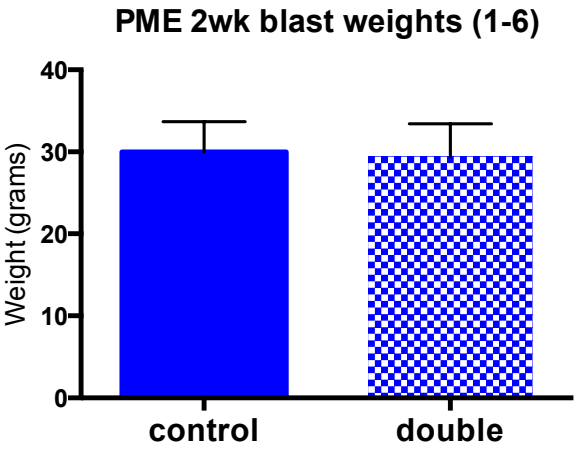


Figure 6: Summary table of behavioral tasks performed in control or PME over expressing animals 2 weeks after sham or shockwave exposure

Behavioral tasks

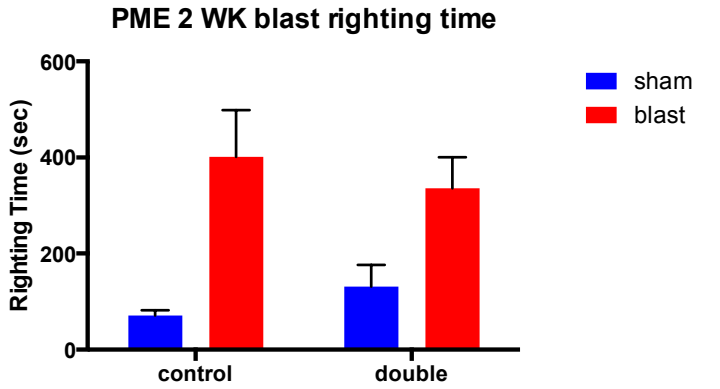
Test/Measure	Function	Figure
Body Weight		6
Righting Time	Acute injury response	7
Rotarod	Motor performance	8-10
Open Field	Motor Performance/Anxiety	11-17
Elevated Plus Maze	Anxiety	18-20
Contextual Fear Conditioning	Anxiety/Cognition	21
Sensory Threshold Assessment	Sensory Perception	22
Radial Arm Water Maze	Cognition	23-24
Visible Platform Water Maze	Vision/Motor Performance/Simple Associative Learning	25-26
Forced Swim Test	Affect/Depression	27
Tail Suspension Test	Affect/Depression	28

Figure 7: Weight in control or PME over expressing animals after sham or shockwave exposure



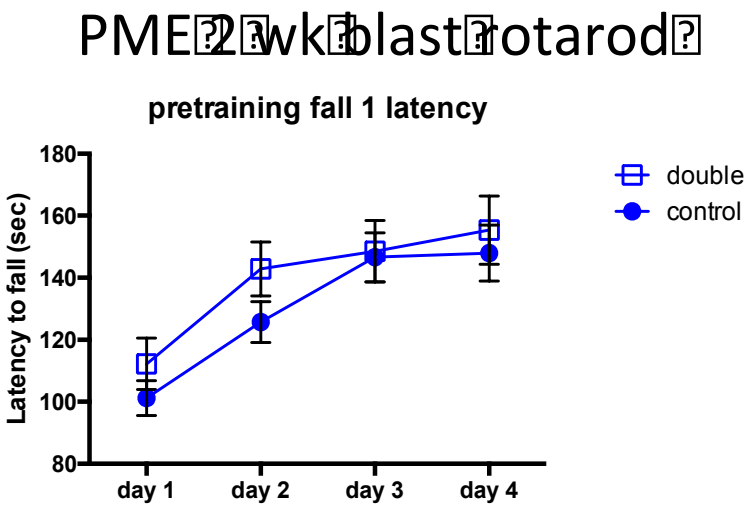
Unpaired t test	
P value	0.5565
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5912 df=63

Figure 8: Righting time in control or PME over expressing animals after sham or shockwave exposure



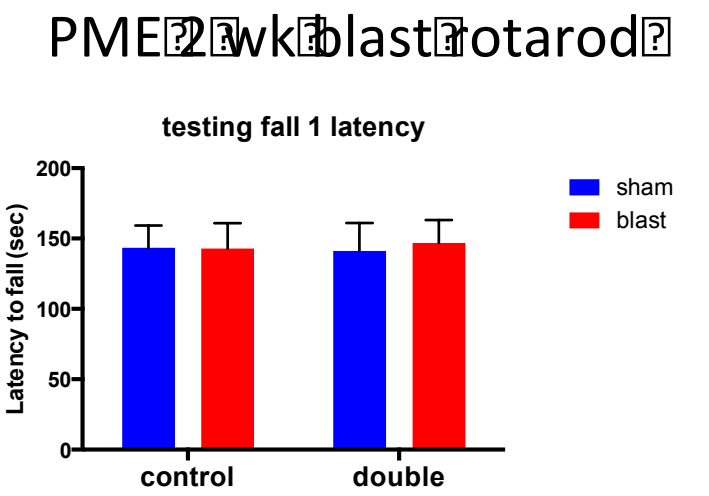
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	63805	1	63805	F (1, 61) = 0.9775	P = 0.3267
GENOTYPE	109.0	1	109.0	F (1, 61) = 0.001671	P = 0.9675
TREATMENT	1.155e+006	1	1.155e+006	F (1, 61) = 17.70	P < 0.0001
Residual	3.982e+006	61	65272		

Figure 9: Pretraining latency during rotarod testing in control or PME over expressing animals before shockwave exposure



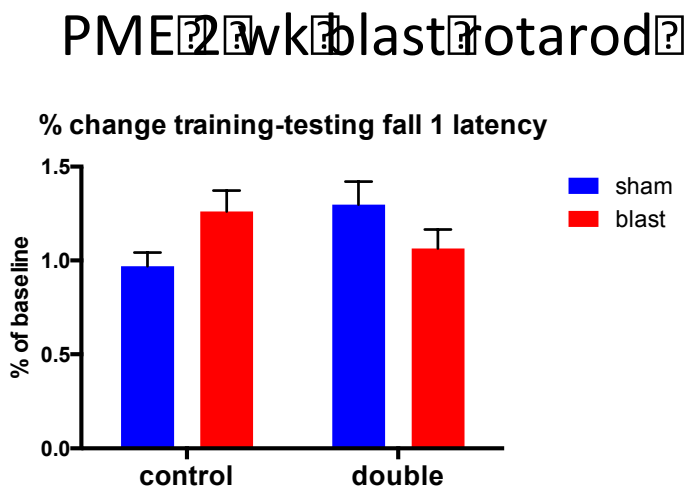
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3999	3	1333	F (3, 520) = 0.2856	P = 0.8358
training day	162669	3	54223	F (3, 520) = 11.62	P < 0.0001
genotype	11626	1	11626	F (1, 520) = 2.490	P = 0.1151
Residual	2.428e+006	520	4668		

Figure 10: Testing fall latency during rotarod testing in control or PME over expressing animals after sham or shockwave exposure



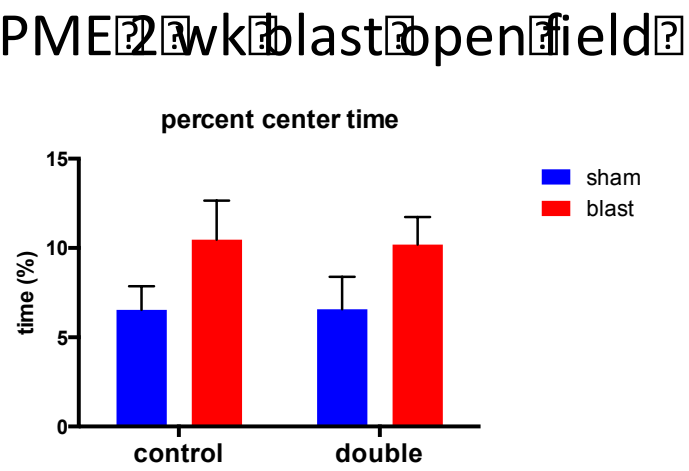
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	143.6	1	143.6	F (1, 59) = 0.03000	P = 0.8631
genotype	13.59	1	13.59	F (1, 59) = 0.002838	P = 0.9577
treatment	96.28	1	96.28	F (1, 59) = 0.02011	P = 0.8877
Residual	282426	59	4787		

Figure 11: Percent training-testing fall latency during rotarod testing in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.080	1	1.080	F (1, 59) = 6.552	P = 0.0131
genotype	0.06725	1	0.06725	F (1, 59) = 0.4081	P = 0.5254
treatment	0.01371	1	0.01371	F (1, 59) = 0.08319	P = 0.7740
Residual	9.723	59	0.1648		

Figure 12: Percent time spent in the center of the arena during open field testing in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.3821	1	0.3821	F (1, 59) = 0.007871	P = 0.92
genotype	0.2043	1	0.2043	F (1, 59) = 0.004208	P = 0.94
treatment	224.1	1	224.1	F (1, 59) = 4.615	P = 0.03
Residual	2864	59	48.55		

Figure 13: Time spent in the periphery and center of the arena during open field testing in control or PME over expressing animals after sham or shockwave exposure

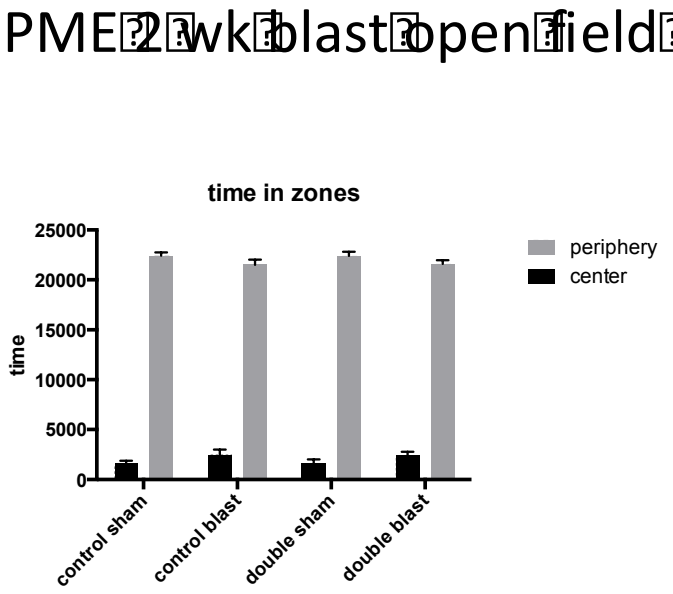
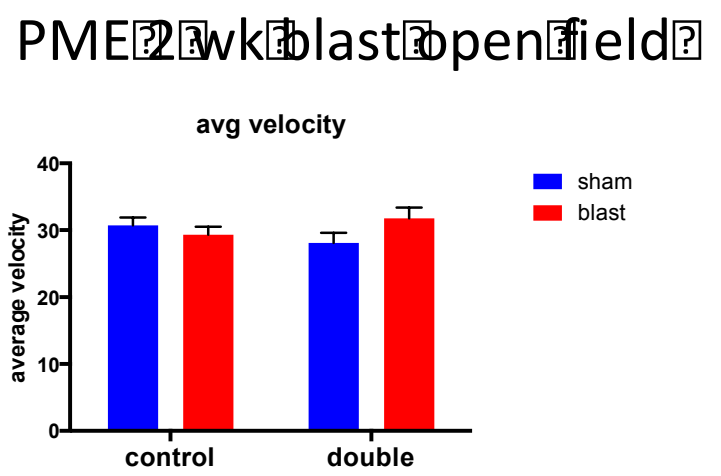
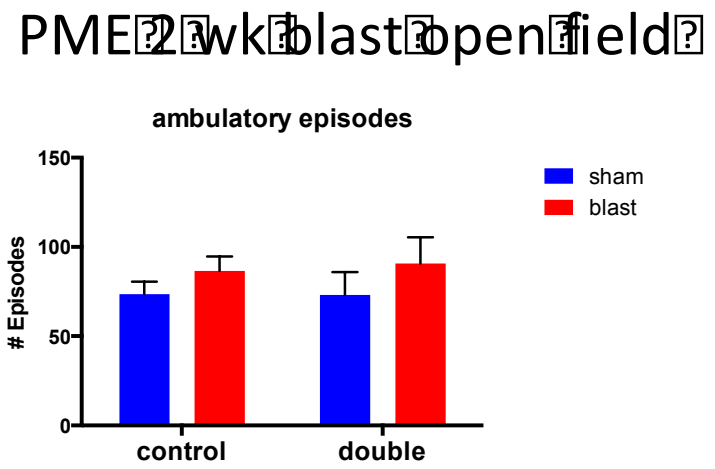


Figure 14: Speed during open field testing in control or PME over expressing animals after sham or shockwave exposure



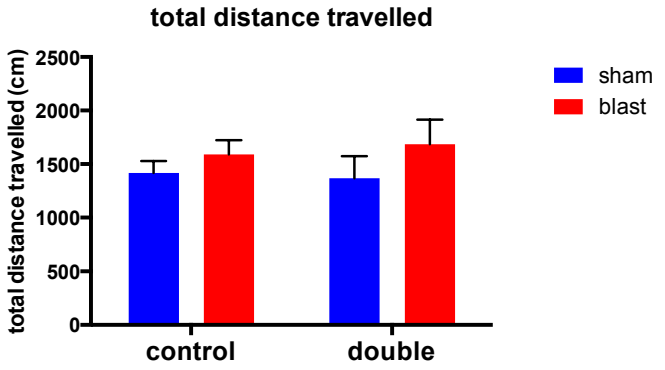
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	100.8	1	100.8	F (1, 59) = 3.403	P = 0.0701
genotype	0.1150	1	0.1150	F (1, 59) = 0.003883	P = 0.9505
treatment	20.85	1	20.85	F (1, 59) = 0.7040	P = 0.4048
Residual	1748	59	29.62		

Figure 15: Number of ambulatory episodes during open field testing in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	85.16	1	85.16	F (1, 59) = 0.04655	P = 0.8299
genotype	50.90	1	50.90	F (1, 59) = 0.02783	P = 0.8681
treatment	3677	1	3677	F (1, 59) = 2.010	P = 0.1615
Residual	107922	59	1829		

Figure 16: Total distance travelled during open field testing in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	81645	1	81645	F (1, 59) = 0.1780	P = 0.6746
genotype	8671	1	8671	F (1, 59) = 0.01891	P = 0.8911
treatment	942303	1	942303	F (1, 59) = 2.055	P = 0.1570
Residual	2.706e+007	59	458640		

Figure 16: Distance travelled in periphery and center during open field testing in control or PME over expressing animals after sham or shockwave exposure

PME 2 wk blast open field

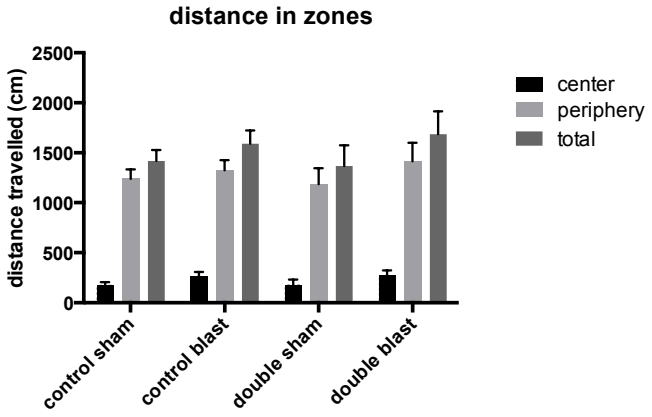
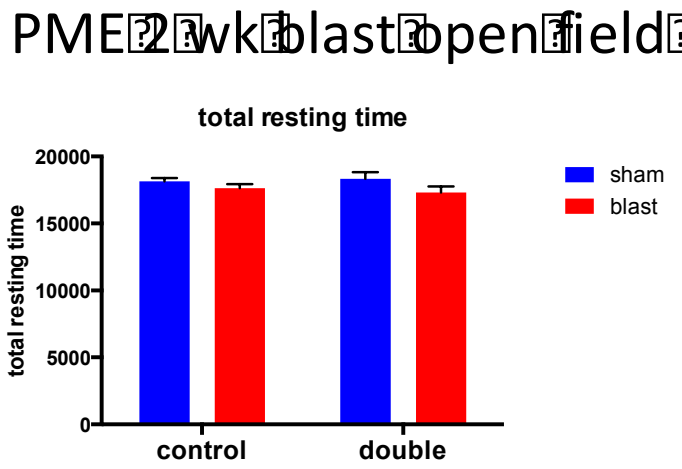
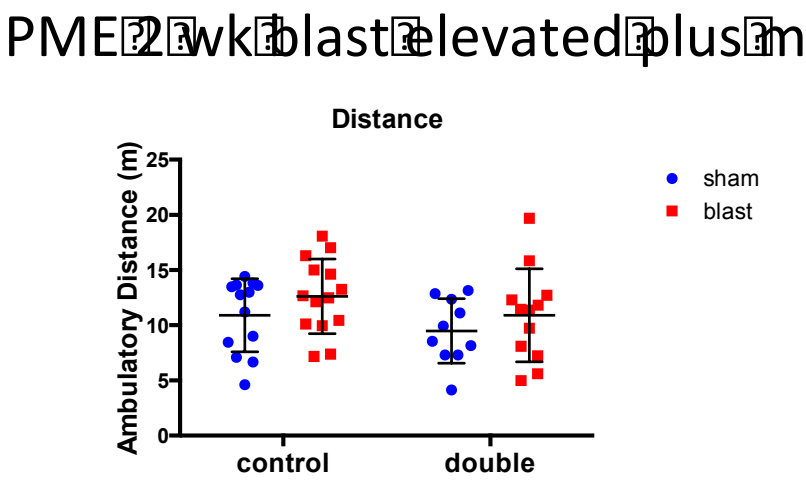


Figure 17: Total resting time during open field testing in control or PME over expressing animals after sham or shockwave exposure



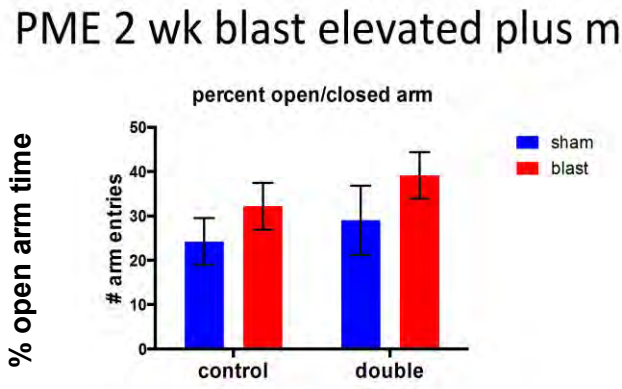
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	989066	1	989066	F (1, 59) = 0.4320	P = 0.5136
genotype	56967	1	56967	F (1, 59) = 0.02488	P = 0.8752
treatment	9.129e+006	1	9.129e+006	F (1, 59) = 3.987	P = 0.0505
Residual	1.351e+008	59	2.290e+006		

Figure 18: Ambulatory distance during elevated plus maze testing in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.2671	1	0.2671	F (1, 45) = 0.02181	P = 0.8833
genotype	29.65	1	29.65	F (1, 45) = 2.421	P = 0.1267
treatment	29.70	1	29.70	F (1, 45) = 2.425	P = 0.1264
Residual	551.0	45	12.25		

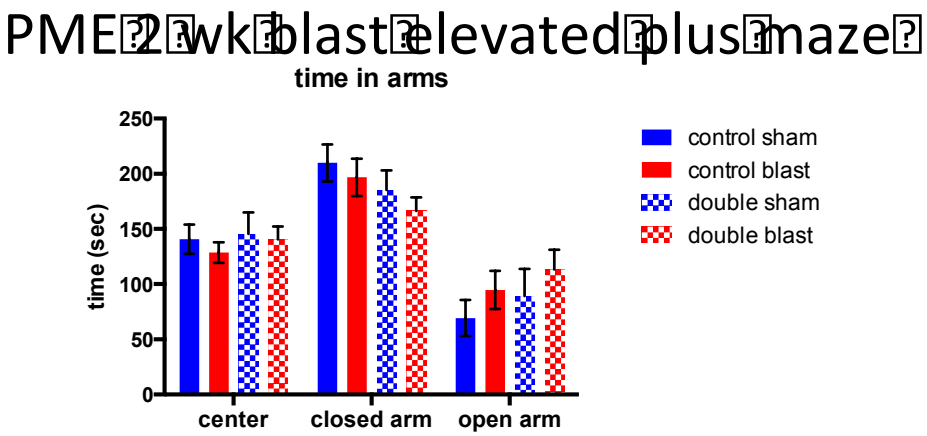
Figure 19: Percent time in open vs. closed arm during elevated plus maze testing in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	14.37	1	14.37	F (1, 45) = 0.03508	P = 0.8523
genotype	414.2	1	414.2	F (1, 45) = 1.011	P = 0.3200
treatment	993.2	1	993.2	F (1, 45) = 2.425	P = 0.1264
Residual	18432	45	409.6		

Figure 15

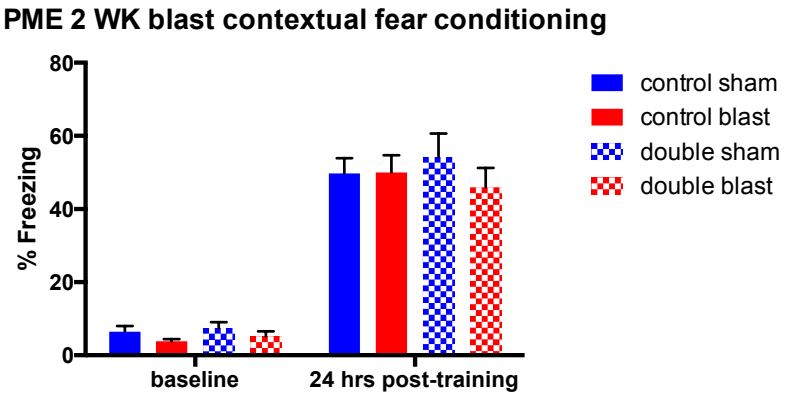
Figure 20: Time spent in the arms during elevated plus maze testing in control or PME over expressing animals after sham or shockwave exposure



Open arm time ANOVA table

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	5.938	1	5.938	F (1, 45) = 0.001386	P = 0.9705
genotype	4413	1	4413	F (1, 45) = 1.030	P = 0.3156
treatment	7324	1	7324	F (1, 45) = 1.709	P = 0.1977
Residual	192836	45	4285		

Figure 21: Percent freezing during contextual fear conditioning in control or PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	176.2	3	58.74	F (3, 59) = 0.2844	P = 0.8364
Time	61284	1	61284	F (1, 59) = 296.8	P < 0.0001
group	447.6	3	149.2	F (3, 59) = 0.6651	P = 0.5768
Subjects (matching)	13237	59	224.4	F (59, 59) = 1.086	P = 0.3756
Residual	12183	59	206.5		

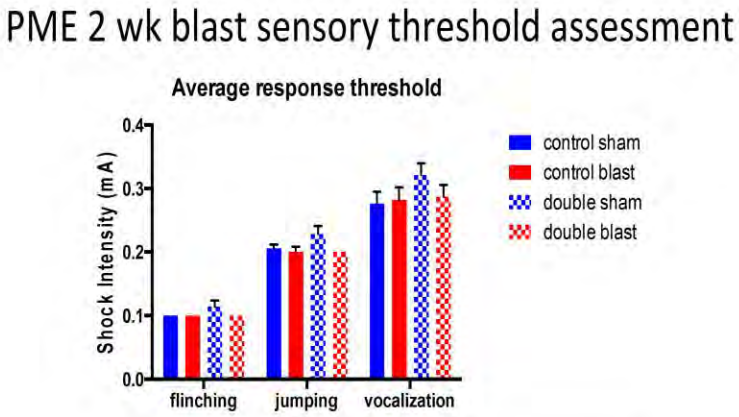
baseline only

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.2663	1	0.2663	F (1, 59) = 0.009656	P = 0.9221
treatment	91.52	1	91.52	F (1, 59) = 3.318	P = 0.0736
genotype	23.39	1	23.39	F (1, 59) = 0.8479	P = 0.3609
Residual	1627	59	27.58		

24 hrs only

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	289.3	1	289.3	F (1, 59) = 0.7174	P = 0.4004
treatment	259.9	1	259.9	F (1, 59) = 0.6446	P = 0.4253
genotype	0.8918	1	0.8918	F (1, 59) = 0.002212	P = 0.9627
Residual	23793	59	403.3		

Figure 22: Sensory threshold assessment in control or PME over expressing animals after sham or shockwave exposure



flinch

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0007980	1	0.0007980	F (1, 59) = 2.746	P = 0.1028
genotype	0.0007980	1	0.0007980	F (1, 59) = 2.746	P = 0.1028
treatment	0.0007980	1	0.0007980	F (1, 59) = 2.746	P = 0.1028
Residual	0.01714	59	0.0002906		

jump

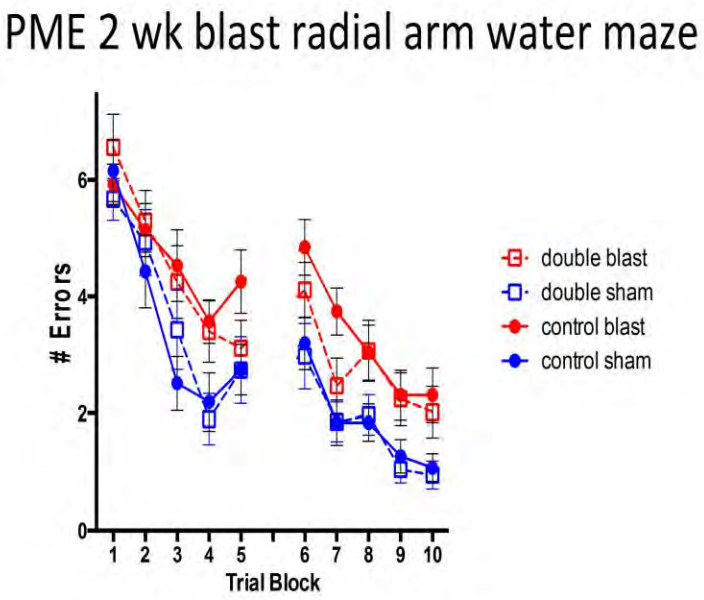
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.002013	1	0.002013	F (1, 59) = 2.048	P = 0.1577
genotype	0.002013	1	0.002013	F (1, 59) = 2.048	P = 0.1577
treatment	0.004642	1	0.004642	F (1, 59) = 4.723	P = 0.0338
Residual	0.05798	59	0.0009828		

vocalization

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.006459	1	0.006459	F (1, 59) = 1.134	P = 0.2914
genotype	0.009493	1	0.009493	F (1, 59) = 1.666	P = 0.2018
treatment	0.003261	1	0.003261	F (1, 59) = 0.5723	P = 0.4524
Residual	0.3362	59	0.005698		

Figure 18

Figure 23: Number of errors during radial arm water maze performance in control or PME over expressing animals after sham or shockwave exposure



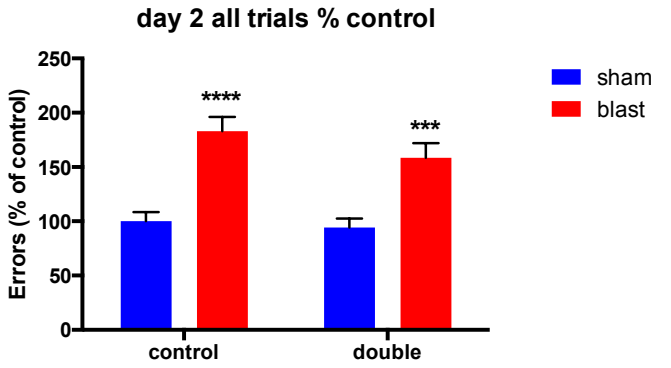
Block 6-10 ANOVA Table

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	10.97	12	0.9140	F (12, 236) = 0.7647	P = 0.6865
trial block	190.1	4	47.51	F (4, 236) = 39.75	P < 0.0001
group	126.7	3	42.23	F (3, 59) = 4.953	P = 0.0039
Subjects (matching)	503.0	59	8.525	F (59, 236) = 7.132	P < 0.0001
Residual	282.1	236	1.195		

Figu

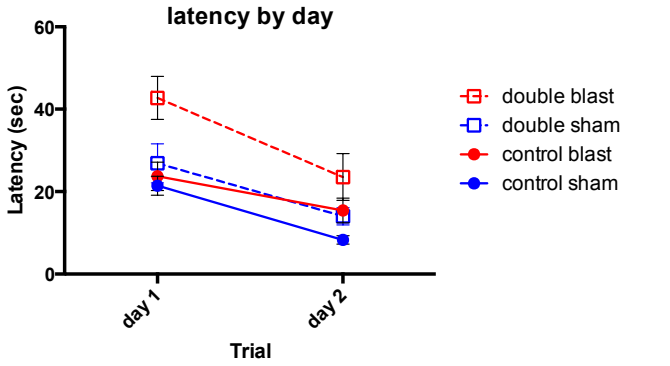
Figure 24: Average number of errors during the second day of radial arm water maze performance in PME over expressing animals after sham or shockwave exposure (percent of controls)

PME 2 wk blast radial arm water maze



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	6989	1	6989	F (1, 311) = 0.7116	P = 0.3995
genotype	17856	1	17856	F (1, 311) = 1.818	P = 0.1785
treatment	423824	1	423824	F (1, 311) = 43.16	P < 0.0001
Residual	3.054e+006	311	9821		

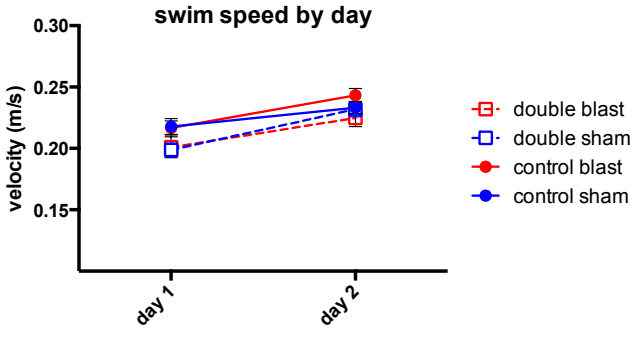
Figure 25: Average latency to the visible platform in controls and PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	475.5	3	158.5	F (3, 59) = 1.017	P = 0.3915
trial	5589	1	5589	F (1, 59) = 35.88	P < 0.0001
group	5717	3	1906	F (3, 59) = 7.220	P = 0.0003
Subjects (matching)	15571	59	263.9	F (59, 59) = 1.694	P = 0.0225
Residual	9191	59	155.8		

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
day 1				
control sham vs. control blast	-2.294	-15.24 to 10.65	No	ns
control sham vs. double sham	-5.445	-19.07 to 8.179	No	ns
control sham vs. double blast	-21.32	-34.69 to -7.948	Yes	***
control blast vs. double sham	-3.151	-16.78 to 10.47	No	ns
control blast vs. double blast	-19.03	-32.40 to -5.654	Yes	**
double sham vs. double blast	-15.88	-29.90 to -1.848	Yes	*
day 2				
control sham vs. control blast	-7.118	-20.07 to 5.831	No	ns
control sham vs. double sham	-5.706	-19.33 to 7.919	No	ns
control sham vs. double blast	-15.24	-28.61 to -1.866	Yes	*
control blast vs. double sham	1.412	-12.21 to 15.04	No	ns
control blast vs. double blast	-8.122	-21.49 to 5.252	No	ns
double sham vs. double blast	-9.533	-23.56 to 4.495	No	ns

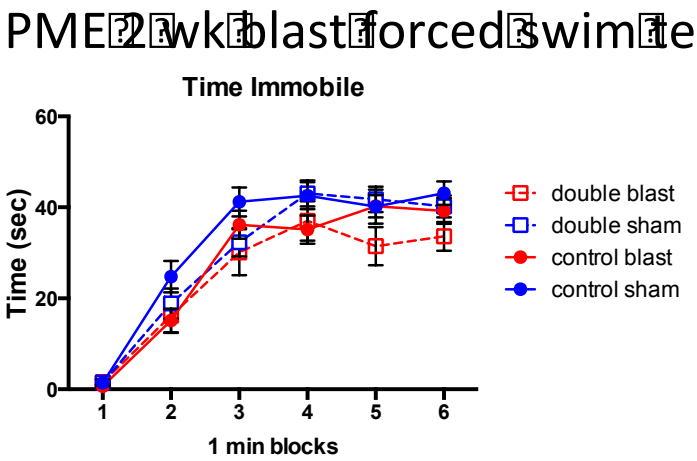
Figure 26: Average speed during visible platform testing in controls and PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.001274	3	0.0004248	F (3, 59) = 1.808	P = 0.1556
trial	0.01919	1	0.01919	F (1, 59) = 81.66	P < 0.0001
group	0.006309	3	0.002103	F (3, 59) = 2.100	P = 0.1099
Subjects (matching)	0.05910	59	0.001002	F (59, 59) = 4.262	P < 0.0001
Residual	0.01387	59	0.0002350		

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
day 1				
control sham vs. control blast	0.001176	-0.02105 to 0.02340	No	ns
control sham vs. double sham	0.01911	-0.004279 to 0.04250	No	ns
control sham vs. double blast	0.01689	-0.006066 to 0.03985	No	ns
control blast vs. double sham	0.01793	-0.005455 to 0.04132	No	ns
control blast vs. double blast	0.01571	-0.007243 to 0.03867	No	ns
double sham vs. double blast	-0.002219	-0.02630 to 0.02186	No	ns
day 2				
control sham vs. control blast	-0.009941	-0.03217 to 0.01229	No	ns
control sham vs. double sham	0.001294	-0.02209 to 0.02468	No	ns
control sham vs. double blast	0.008627	-0.01433 to 0.03158	No	ns
control blast vs. double sham	0.01124	-0.01215 to 0.03462	No	ns
control blast vs. double blast	0.01857	-0.004388 to 0.04152	No	ns
double sham vs. double blast	0.007333	-0.01675 to 0.03142	No	ns

Figure 27: Time spent immobile during the forced swim test in controls and PME over expressing animals after sham or shockwave exposure

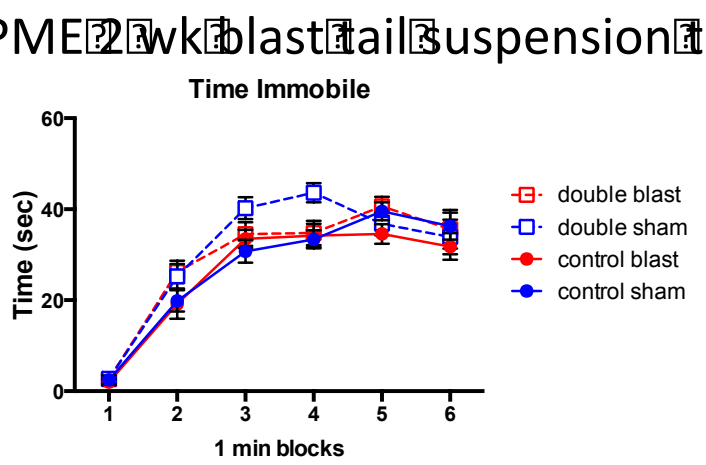


ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1920	15	128.0	F (15, 295) = 1.209	P = 0.2632
Time	75525	5	15105	F (5, 295) = 142.7	P < 0.0001
group	2654	3	884.5	F (3, 59) = 2.655	P = 0.0567
Subjects (matching)	19659	59	333.2	F (59, 295) = 3.148	P < 0.0001
Residual	31223	295	105.8		

Tukey's multiple comparisons test

	1	2	3	4	5	6
control sham vs. control blast	ns	ns	ns	ns	ns	ns
control sham vs. double sham	ns	ns	ns	ns	ns	ns
control sham vs. double blast	ns	ns	*	ns	ns	ns
control blast vs. double sham	ns	ns	ns	ns	ns	ns
control blast vs. double blast	ns	ns	ns	ns	ns	ns
double sham vs. double blast	ns	ns	ns	ns	ns	ns

Figure 28: Time spent immobile during tail suspension test in controls and PME over expressing animals after sham or shockwave exposure



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1767	15	117.8	F (15, 295) = 1.635	P = 0.0640
Time	58683	5	11737	F (5, 295) = 162.8	P < 0.0001
group	1152	3	384.0	F (3, 59) = 1.852	P = 0.1476
Subjects (matching)	12233	59	207.3	F (59, 295) = 2.876	P < 0.0001
Residual	21264	295	72.08		

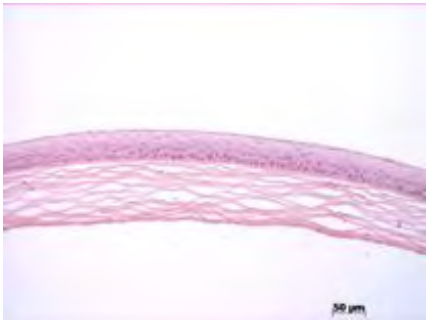
Tukey's multiple comparisons test

	1	2	3	4	5	6
control sham vs. control blast	ns	ns	ns	ns	ns	ns
control sham vs. double sham	ns	ns	*	*	ns	ns
control sham vs. double blast	ns	ns	ns	ns	ns	ns
control blast vs. double sham	ns	ns	ns	*	ns	ns
control blast vs. double blast	ns	ns	ns	ns	ns	ns
double sham vs. double blast	ns	ns	ns	ns	ns	ns

Blast Exposed Eye

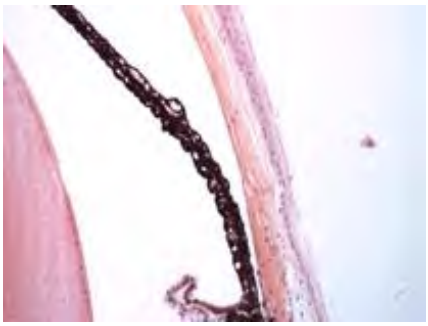
Abnormal Findings: Hyphema, vitreous hemorrhage [MP:0006202], retinal degeneration [MP:0001326], retinal hemorrhage [MP:0006185].

EYE Phenotype



Cornea:

1/ 6. There was hyphema in one eye. Otherwise there was a normal corneal epithelium, stroma, and endothelium.



Anterior chamber:

6/ 6. The anterior chamber was of normal depth without cells, and the angle appeared open.

Iris:

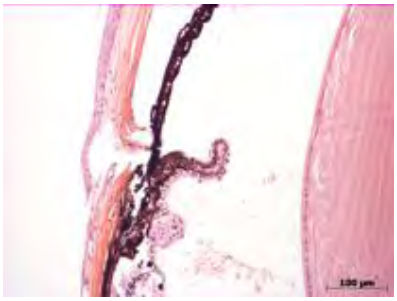
6/ 6. The iris showed normal pigmentation without rubeosis or pupillary membranes.

Figure 30: histology on eyes from blast exposed mice.

Blast Exposed Eye

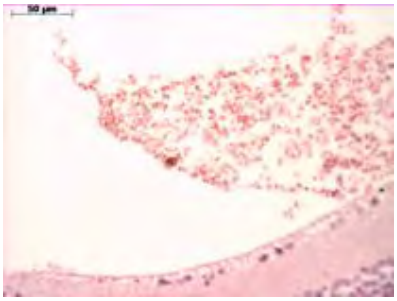
Lens:

6/ 6. No cataract was observed.



Ciliary body:

6/ 6. Normal stroma, pigmented and nonpigmented layers were present along with cilia.

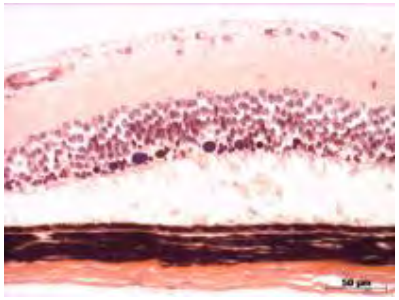


Vitreous:

3/ 6. There was posterior vitreous detachment, vitreous hemorrhage, and rare inflammatory cells.

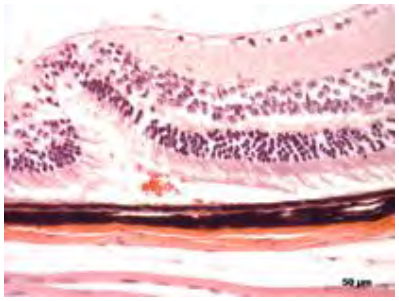
Figure 31: histology on eyes from blast exposed mice.

Blast Exposed Eye



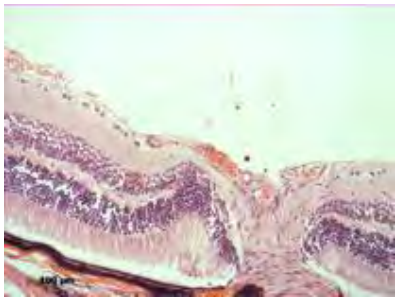
Retina:

3/ 6. There were foci of photoreceptor degeneration and pigmentary changes. There was also subretinal hemorrhage (see image below)



Retinal pigment epithelium and Choroid:

6/ 6. Normal pigmentation. Bruch's membrane is intact. No neovascular membranes were noted.



Optic Nerve:

6/ 6. The nerve is normal.

Methods. 6 eyes from 3 male mice were enucleated by blunt dissection and fixed. Pupil-optic nerve sections were processed with hematoxylin and eosin, and standard images were captured under light microscopy for review.